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Cross- reactions of the monospecific Hydatid cyst sera with some helminthes antigens in sheep

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Abstract

Echinococcus Granulosus **is the causative agent of Hydatidosis, which is an important zoonotic disease with worldwide distribution. This study aimed to identify the cross- reaction of the hydatid cyst fluid antisera with antigens of *Fasciola hepatica*, *Taenia hydatigena* (*CysticercusTenuicollis*), *T. thysaniezia*, and *Moniezia expansa* by application of *Immunoblot* test and dot ELISA techniques. Mono- specific serum was obtained by immunizing rabbits against the 54, 38 and 27 KDa antigens individually; and Sera were collected from rabbits before injecting the antigens and used as negative control.**

The results showed that, serum induced by bands which have molecular weight 54 KDa antigen reacted with antigens of *CysticercusTenuicollis* and *Moniezia expansa*, while, serum induced by band 38 KDa antigen strongly reacted with antigens of *T.thysaniesia* and *Fasciola Hepatica*. On the other hand, serum induced by band 27 KDa antigen reacted with antigens of *T. thysaniesia* and *Moniezia Expansa*. However, there was no cross - reaction between antigens of *Fasciola hepatica* and *Cysticercus tenuicoillis* with antiserum band 27 KDa.

It is concluded that, there is an antigenic relation between *Hydatid* cyst fluid and different studied parasites. It is proposed that subunit with molecular weight 38 KDa is satisfactory antigen would be used in serodiagnosis for detection of *hydatidosis* antibodies in Syrian sheep.

Keywords: Hydatid cyst antigens, cross- reaction, sheep.

Introduction

Hydatidosis is a cosmopolitan distribution disease found in a wide variety of ungulates and humans. It is caused by *Taenia* tapeworm; *Echinocoucus granulosus* spp. Which considered as one of the neglected seven diseases in the world (Soulsby, 1987; Eckert *et al.*, 2002 and WHO, 2006). In Syria hydatidosis have a negative impact on health and economic problem of endemic nature. The prevalence of hydatidosis in this country was found to be 46.4% in older sheep (over one year of age), while infection rate of

Cysticercus tenuicollis alone reached 9.69% (AL-Khaled, 2001; Al- Yasin and Guerouali, 2011).

In humans, the early diagnosis of hydatidosis is very important for successful treatment. Therefore, cyst structures are identified by various diagnostic imaging techniques such as ultra sonography, computed tomography, and X-rays. Newly formed

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cysts can be confirmed by radiography and ultrasound (Von Sinner, 1991; Schmitt *et al*., 1997 and Moro and Schant, 2008). Indirect Haemagglutination (IHA), indirect Immunofluoresence antibody test (IFAT), Immunoelectrophoresis (CIEP), Double diffusion test (DDT), and enzyme linked sorbent assay (ELISA) were employed for the early antibody detection of *Echinococcosis* (Eckert and Deplazes, 2004).

The most common antigenic sources used for the immunodiagnosis of human hydatidosis are hydatid cyst fluid, and somatic excretory/secretory products from *protoscoleces* or adults of *Echinococcus granulosus* (Carmena *et al*., 2006). The concentrations of protein and antigens 4 and 5 in hydatid fluids were assessed in sheep hepatic cyst of 0.088 mg/ml, and in pulmonary cysts of 0.0089 mg/ml, and the concentration of total protein was 5.8% (Musiani *et al*., 1978). However, the serodiagnostic tests have some disadvantages particularly cross-reactions with *Taenia* spp. or with other helminthes infections, leading to false positive results such as with *Trichostrongylidae, Paramphistomum spp., Ticharis spp., Fasciola spp., Dicrocoelium dendriticum,* and *Cysticercus tenuicollis.* Most cross-reactions were reported with the bands in the molecular weight of 98, 68, 58, 38 KDa, according to the following proportion: 100%, 85%, 55%, 70%, respectively (Burgu *et al*., 2000).

There were considerable differences in the sensitivity and specificity of serodiagnostic tests for hydatidosis in variant preparations of antigens and standardizations of different techniques used among some studies. The sensitivity of B-ELISA was found to be 89%, and for subunits 8/12, 16 KDa immunoblot was 80%. Meanwhile, the specificity of the AgB-ELISA was 98% with subunits 8/12, 16 KDa immunoblot was 100%. According to Haniloo *et al* (2005), the crude antigens had sensitivity and specificity of 94%, 83% respectively. Also, Siavashi *et al* (2005) obtained that high sensitivity and specificity up to 100% and 98.75% by using dot- ELISA, and 92.22% and 98.75% by sandwich ELISA, respectively which tested by using the crude antigens from lung and liver cysts from sheep and human sera. However, cross-reactions were observed with Fasciolosis in both techniques. In addition, cross-reactions were reported with sera from patients with *Cysticercosis*, *Filariasis*, *Toxocariasis*, *Trichinosis*, Visceral larva migrations, and liver cirrhosis, by Dot- ELISA using crude antigen from sheep hydatid fluid (Swarna and Parija, 2008).

Hydatid proteins formed cross-reactions with heterologous sera collected from patients harboring Ascarid, poly parasitosis, *Bilharzia, Leishmania, Toxoplasma*, cancer and *Fasciola* (Kaderi, 1991 and Hadighi *et al*., 2003), while cross- reaction antigen fractions with molecular weight under 24 KDa was not detected (Kaderi, 1991), and Hadighi

et al (2003) obtained sensitive and specific differences of serodiagnostic tests to be 97.1%, and 98.5% respectively by Dot- ELISA using antigen B from sheep hydatid fluids, whereas they observed one serum out of 12 cases has been cross reacted with patient serum with Fascilosis. Whereas, sera collected from patients with Amoebiosis, Leishmaniosis, Toxoca-riosis, and Toxoplasmosis didn't have cross- react. The band with molecular weight 45KDa was responsible for cross- reactivity among crude antigens of *Cysticercus ovis*, *Cysticercus bovis*, *Cysticercus taenuicollis*, and may be with other metacestodes (Kandil *et al*., 2004).

Serodaignosis of cystic *echincoccosis* in sheep -the main intermediate host of *Echinococcus granulosus*- has been carried out by using *hydatid* fluids and crude parasite antigens that have poor antibody responses to the infection and cross-reactions with other *Cysticercus taenuicollis* and *T.*ovis (Kittelbreger *et al.,* 2002).

Epidemiologic data provided that Syrian sheep have high prevalence of *T. moneizia* , *T.hydatigena* and *T. thysaneisia*, but low prevalence in *Fasciola* hepatica (Al -Khaled, 1999 and Al -Yasin and Al -Khaled, 2008).

The objective of this study was to identify cross –reactivity of Hydatid cyst fluid antigens subunits with helminthes of sheep *(Cysticercus tenuicollis*, *T. Avitellina*, *Fasciola Hepatica Moniezia Expansa*, and *T.thysaneisa*) in order to obtain satisfactory antigens with less cross- reaction, for using in seroepide-miologic surveys.

Materials and Methods

Preparing of Hydatid cyst fluid (HCF) antigen:

Antigen was prepared from the viable Hydatid cysts of hepatic origin obtained from naturally infected sheep, at the local slaughterhouse. Briefly, hydatid cyst fluid was collected aseptically from the cysts and some drops of Sodium Azide 1%. Then centrifuged at 3000 g for 30 min. to settle the *protoscolices.* The supernatant was taken as crude antigen, and then the solute proteins were precipitated nor Albumin by adding NH_3SO_4 in rate of 40 % (w/v). Then stirred on magnetic mixing at +4 °C over night, then centrifuged at 10000 g at 60 min at +4°C. The supernatant was discarded out and the pellet was taken and dissolved in Phosphate Buffered Saline (PBS), pH=7.2. Then solution was dialyzed against distilled water at +4°C for 56 hours. This fluid was then re-dialyzed against Polyethylenglycol 1500 to concentrate the antigens. Dispensed in small aliquots and stored as HPR antigen at -20 °C until further use. The protein concentration was estimated by using UV^{280} with bovine serum albumin as a reference standard.

Preparing worms antigens:

Fasciola hepatica, *T.hydatigena (Cysticercus tenuicollis*), *T.Thysaniezia*, and *Moniezia expansa*, were collected from the local abattoirs, then washed three times in PBS, pH=7.2, hereafter

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incubated for 2 hours at 37 °C, then washed by PBS, and some pieces from the strobila and were homogenized, then centrifuged at 10000 g for 30 min, and the supernatant transferred into beaker. Ammonium Sulphate was added in rate of 40% (w/v) on magnetic mixer at +4°C overnight then some drops of sodium azide were added, and centrifuged at 10000 g for one hour, then precipitated pellet was dissolved in PBS, and transferred into dialysis sac against the distilled water for 3 days at +4°C. Water was changed at intervals of 8 hours, then *dispensed in small* aliquots in 2 *ml cryoprotected vials* and preserved at -20 °C. The *Hydatigena* liquid was treated as the protocol aforementioned in *Hydatid cyst* liquid case.

SDS-PAGE:

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method as described by Smith (1994) using 12.5% slab gels under reducing conditions by using PROTEAN-II XI cell (Bio-Rad). Briefly, the mixture of four volumes of each antigen, HCF-Ag (1 mg protein/ml), *Fasciola hepatica*, *Cysticercus tenuicollis*, *T.Thysaniezia*, and *Moniezia expansa*, were mixed with one volume of sample buffer (4x). High molecular weight marker (SERVA, Cat.No.392071/P080257) was used as standard. Running gel applied at constant current (50V) overnight.

Preparation of Hyperimmune rabbit antisera:

Production of antibodies by using proteins in the gel bands was followed as described by Amero *et al.*, (1994). Briefly, gel plate was stained for 30 min in Coomassie blue R250 solution, and partially destained using gently agitation in several times, then immersed in 2% Glutaraldehyde for 60 min, the continued destaining steps till the gel was completely transparence. Then the gel was washed in the deionized water in several times, then cut out the interested bands (27, 38 and 54KDa) and separately pulverized by passing the pieces of gel through sterile syringe.

Six white rabbits were used at 500 g body weight (two rabbits for each antigen), and quarantined for 10 days in individual cages. Antigens 54, 38 and 27 were diluted to 1.0 ml with sterile PBS pH, 7.2, and mixed with an equal volume of complete Freund's Adjuvant for the first immunization given S/C, and mixed with Incomplete Freund's Adjuvant (IFA) for the second immunization 3 weeks after the first injection. Two sub sequent immunization was carried out as for the 1 day; with 14 days intervals, the rabbit was ear bled and the blood was clotted at room temperature for 30 minutes and then at 4C° for 4hours. The collected sera were stored at -20C°.

Dot-ELISA:

The Dot-ELISA was used as described by Swara and Parija (2008). Briefly, three different antigenic subunits of *Hydatid* cyst fluid (27, 38, and 54 antigens) were involved in the detection of monospecific IgG antibodies against subunits 27, 28, and 54 antigens. The optimization dilutions of serum was 1:2000 and goat anti rabbit IgG- conjugated HRP 1:10000, (KPL, No.074-1506), and used the substrate 3, 3', 5, 5' Tetramethylbenzidine (TMB)(sigma, No.T0565) to show the

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reaction. Pieces coated antigens were blocked by PBS-T added 3% NFM.

Immunoblotting:

The separated proteins (the studied worms antigens) on the gel plate using PROTEAN-II XI cell that aforementioned, were transferred onto nitrocellulose membrane using Transfer apparatus: Trans –blot cell, Bio-Rad, according to described by Page and Thorpe(2002) for 90 minutes at 100V. The reactions on membranes were detected according to Rybicki and Purves (1996). Briefly, membrane was incubated for one hour at room temperature (RT) in Blocking Solution (PBST-5%NFM) containing phosphate buffered saline in addition to 0.05% Tween-20 and 5% nonfat milk powder (NFM). Then incubated in hyperimmune antisera 1:2000 dilutions in PBST - 3%NFM for 60 min at RT. After washing with PBST, the membrane was incubated in Anti sheep IgG-HRP conjugates (Kpl, No. 074) diluted 1:10000 in PBST-1%NFM for 60 min at RT. The membrane was washed with PBST, then visualization the reaction by incubating membrane in substrate TMB (Sigma).

Results:

a- Dot –blot: The hyperimmune antisera against subunit 54 KDa was reacted with whole crude antigens of *Moniezia expansa* and *C. tenuicollis*, while the crude antigens of *T. thysaniesa* and *Fasciola hepatica* were not reacted (Table 1, Figure3). On the other hand, the hyperimmune antisera against subunit 38 KDa was reacted with whole crude antigens of *T. thysaniesa* and *Fasciola hepatica*, and reacted weakly with *Moniezia expansa*, but didn't have reaction with antigens of *C. tenuicollis*. The hyperimmune antisera against subunit 27 KDa cross-reacted only with whole crude antigens of *Moniezia expansa* and *T. thysanies*.

b- **Immunoblotting:**

The results of the immunoblotting of the hyperimmune antisera against subunit 54 showed that the cross-reaction detected with *Cysticercous tenuicollis* (Subunit 54 KDa), and *Moniezia expansa* antigens (Subunit 49KDa). While on contrary, there are no cross-reactions with each of *Fasciola hepatica* and *T.thysaniesia* (Fig. 2).

Subunits 38 showed strong cross-reactions against *Fasciola hepatica* in the both bands 57 and 43 KDa, and

with *T.thysaniesa* in the four bands (43, 45, 59, 84 KDa), while it cross-reacted weakly with *Moniezia expansa* antigens in only one band (25KDa). On the other hand, there is no reaction registered with *C.tenuicollis* antigens (Fig. 3).

The subunit 27 showed strong cross-reactions in five bands with antigens of *Moniezia expansa* (25, 42, 64, 67, 72 KDa), as well as with antigens of *T.thysaniesa* in six bands (43, 67, 84, 98, 130, 173 KDa) and the band 98 KDa had the clearest reaction, while, there is negative cross-reactions with each of *Fasciola hepatica* and C.*tenuicollis* (Fig. 4).

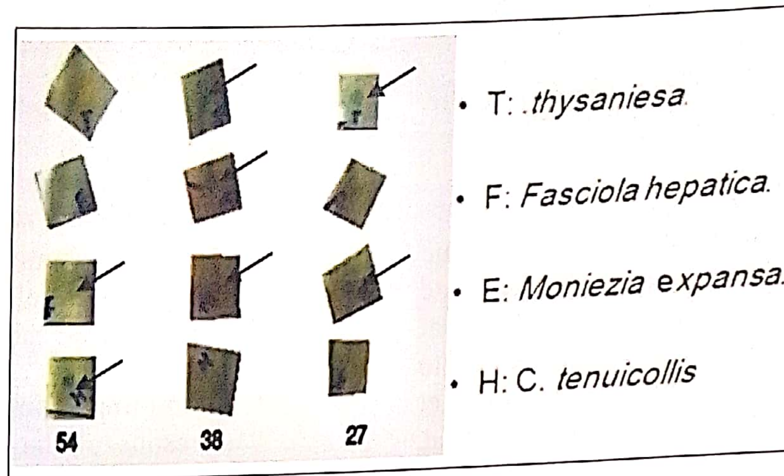

Fig.(3): Dot-ELISA of the crude antigens with monospesific sera against subunits 27, 38, and 54 KDa with each of; T: T.*thysaniesa*. F: *Fasciola hepatica*. E: *Moniezia expansa*. and H: C. *tenuicollis*.

Table (1): Results of across reactions between monospecific sera to *hydatid cyst* fluid antigens subunits and studying worms antigens.

Name of parasite	Hyperimmun antisera		
	Band 27	Band 38	Band 54
C.tenuicollis	Non	Non	strong
M.expansa	Strong	weak	strong
T.thysaniesia	Strong	strong	Non
Fasciola Hepatica	Non	strong	Non

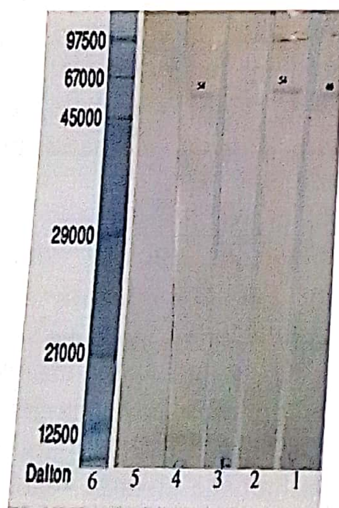

Fig 2. B and 54:
1: ***M.expansa.*** **2:** ***Hydatid*** **cyst. 3:** ***Fasciola hepatica.***
4: ***C.tenuicollis.***
5: ***T.thysaniesia.***
6: :Marker protein

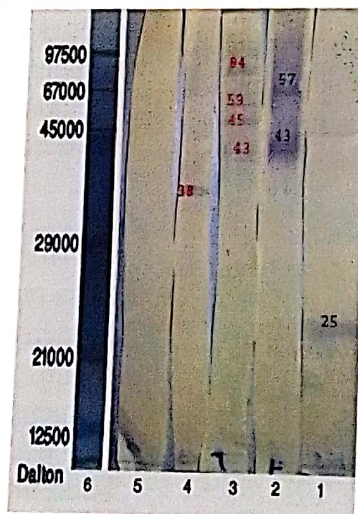

Fig 3. B ands 38:
1: ***M.expansa.***
2: ***Fasciola hepatica.***
3:***T. thysaniesia.***
4: ***Hydatid*** **Cyst.**
5: ***C. tenuicollis.***
6: :Marker protein

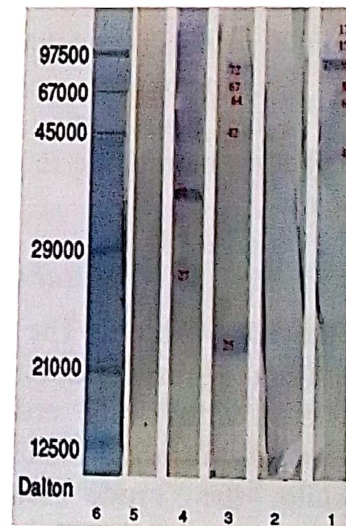

Fig4. b and 27:
1: T. ***thysaniesia.***
2: C. ***tenuicollis.***
3: M. ***expansa.***
4: ***Hydatid*** **cyst.**
5: ***Fasciola hepatica.***
6:Marker protein

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Discussion

Serodiagnosis of *hydatidosis* in sheep is an important tool for an early diagnosis of infection and subsequent treatment. However, the main obstacle in the immunodiagnosis tests of hydatidosis is cross reactions with other parasites, which gave arise false positives and reduce the efficacy of the test. Immunological assays for the diagnosis of *E. granulosus* metacestodes in intermediate aberrant hosts were less sensitive and specific than that for humans (Meslin and Pawlowski, 2002). Accurate immunodiagnosis of the infection requires highly specific and sensitive antigens to be used in immunodiagnostic assays.

In the present study, the cross-reactivity of the mono-specific sera against hepatic *hydatid* fluids antigenic the three subunits; 27, 38, and 54 KDa was studied. The results of Dot-ELISA test showed that subunit 54KDa across-reacted with whole crude antigens of *Cysticercous tenuicollis* and *Moniezia expansa*. On the other hand, the subunit 38 showed strong cross-reaction against whole antigens of *Fasciola hepatica*, and *T.thysaniesia*, while weakly across-reaction with whole antigens of *Moniezia expansa*. Also, the hyperimmune antisera against subunit 27 cross-reacted strongly with antigens prepared from *Moniezia expansa* and *T. thysaniesa*.

The results of the immunoblotting showed that the hyperimmune antisera of subunit 54 cross-reacted with *Cysticercous tenuicollis* (band 54 KDa), and *Moniezia expansa* antigens in the band 49KDa. While on contrary, there are negative cross-reactions with *Fasciola hepatica* and *T.thysaniesia* antigens (Figure2). Also, Subunits 38 showed strong cross-reactions against *Fasciola hepatica* antigens in the both bands 57 and 43 KDa, and with *T.thysaniesa* antigens in the four bands (43, 45,59,84 KDa), while it had cross- reacted weakly with *Moniezia expansa* antigens in only one band (25KDa). Meanwhile, negative reactions with *C.tenuicollis* antigens (Figure 3). While, the subunit 27 showed strong cross-reactions against antigens of *Moniezia expansa* in five bands (25, 42, 64, 67, 72 KDa). As well as with *T.thysaniesa* in six bands (43, 67, 84, 98,130,173 KDa) (Fig. 4).

In general, the results of western blotting by using fraction pure antigens were corresponded with that obtained of Dot-EILSA. Sabry(2007) reported that the subunit 32-38 KDa has only got cross –reactions with *Moniezia expansa* and not reacted with *Fasciola hepatica,* while the subunit 16-18 KDa was reacted with *Moniezia expansa* and *Fasciola hepatic*. These differences may be due to geography region, type of animal and the strains .

Several studies obtained cross-reactions with hydatid cysts, Haniloo *et al.*, (2005) showed that there are across- reactions with both subunits 27 and 24, while subunits 38 was cross-reacted with Toxocara. Burgu *et al.,* (2000) had similar results with four subunits; 38, 58, 68, 98 KDa, that cross-reacted with *Fasciola spp., Dicrocoelium dendriticum, Cysticercus tenuicollis, Trichostrongylidae, Paramphistomum spp. Tichuris spp.* Swarna and Parija (2008) obtained an antigenic relations with some helminthes, particularly *Fasciola spp., Dicrocoelium dendriticum, Cysticercusis* also *liver cirrhosis* and *visceral larva* migrations and *cancer.* However, Hadighi *et al.*, (2003) reported only one case that reacted with *Fasciola hepatica.* Additionally, Siavashi *et al* (2005) observed that there is cross-reaction with *Fasciolosis* in both sandwich ELISA and dot- ELISA techniques. In contrast, Kaderi (1991) found that antigens of subunits 24 KDa, had negative cross- reaction with the aforementioned parasites. The previous studies indicated that the three antigenic subunits 8, 12 and 16 were the most specific (Kaderi, 1991; Haniloo *et al.*, 2005). All these discrepancies may be attributed to the presence of variant strains of *echinococcus* and different sources and preparation techniques of the antigens and standardizations. Additionally, variations in host species of the above mentioned parasites

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could be play a role in the specificity of diagnostic techniques.

Some studies were based on sera collected from patients as control positive reference (Burgu *et al.*, 2000; Siavashi *et al.*, 2005; Haniloo *et al.*, 2005; Swarna and Parija, 2008), and therefore some of the obtained results may not be reliable. Based on the epidemiologic data in Syria, *Taenia moneizia* and *T. hydatigena*, and T. *thysaneisia*, but not *Fasciola hepatica* are prevailing in Sheep (Al-Khaled, 1999; Al-Yasin and Al- Khaled, 2008). which could be proposed that the subunits 38 KDa may be a satisfactory antigen to be used for detection of *hydatidosis* antibody in Syrian sheep.

In conclusion there is a relationship between antigens of the *Hydatid* cyst fluid and antigens of other parasites. Further studies were needed to focus on the cross-reactions of *Hydatid* cysts with another helminthes of sheep and select the highest specific and sensitive antigen for serodiagnosis of this important disease. Additionally, there are more researches needed to examining other subunits antigenic bands in weight especially less than 27 KDa of *Hydatid* cyst fluids.

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الملخص العربي

تعد الدودة الشريطية المشوكة الحبيبية المسبب لداء الكيسات العدارية، وهو مرض مشترك هام واسع الانتشار في العالم. هدفت هذه الدراسة إلى تحديد التفاعلات التصالبية بين أمصال سائل الكيسات العدارية و مستضدات الدودة الكبدية والكيسة دقيقة الرقبة والشريطية التيسانيزية والمونيزية اكسبانزا باستعمال طريقة التبصيم المناعي وتقنية الاليزا النقطية. تم الحصول على الأمصال وحيدة النوعية من خلال تمنيع الأرانب ضد مستضدات سائل الكيسات العدارية 54 و83 و27 كيلو دالتون بشكل مستقل، جمعت الأمصال من الارانب قبل التمنيع واستعملت كشاهد سلبي.

أظهرت النتائج أن المصل وحيدة النوعية للمستضد 54 كيلودالتون تفاعل مع مستضدات الكيسة دقيقة الرقبة والمونيزية اكسبانزا، في حين تفاعل المصل وحيدة النوعية للمستضد 38 كيلو دالتون بشكل قوي مع مستضدات الشريطية تيسانيزية والمتورقة الكبدية. ومن جهة أخرى تفاعل المصل وحيد النوعية للمستضد 27 كيلو دالتون مع مستضدات الشريطية التيسانيزية والمونيزية اكسبانزا. على أيّة حال، لم تحصل تفاعلات تصالبية بين المصل وحيدة النوعية للجزيئة 27 كيلو دالتون مع مستضدات المتورقة الكبدية والشريطية دقيقة الرقبة.

يستنتج من هذه الدراسة أنه يوجد قرابة مستضدية بين سائل الكيسات العدارية ومختلف الطفيليات المدروسة. ان من المقترح ان الوحدة ذات الوزن الجزيئي 38 كيلو دالتون تشكل مستضد مقبول حيث يمكن أن تستعمل في التشخيص المصلي للكشف عن أضداد الكيسات العدارية في الأغنام السورية.

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